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Characterization of an 84 kDa protein inducing an immediate hypersensitivity reaction in rabbits sensitized to *Haemaphysalis longicornis* ticks

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Abstract

An immunogenic 84 kDa protein was isolated and purified from whole tick extracts of *Haemaphysalis longicornis* larvae by a combination of ion exchange, reverse phase and hydrophobic interaction chromatographies. The protein, when injected intradermally into rabbits exposed to repeated tick feeding, induces an immediate cutaneous hypersensitivity reaction. It has been purified to homogeneity as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis and silver staining. Amino acid sequences for two peptides derived from proteolytic cleavage of p84 were scanned against known proteins on the SWISS-PROT database. A 7 residue motif, ISGWGNT present in one of the two peptides appeared conserved in both vertebrate and invertebrate trypsin-like serine proteinases, while another 7 amino acid motif, HVPAGQI present in the second peptide showed homology to an *Escherichia coli* ATP-binding protein. We have discussed our findings in relation to isolation and characterization of target antigens for tick vaccine candidates.

Keywords: Cutaneous hypersensitivity; Ixodid tick; Serine protease; *Haemaphysalis longicornis*

1. Introduction

Ticks represent one of the most important groups of arthropods that affect animal and human health. Among the external parasites, ticks produce the greatest economic loss in livestock production world-wide [1]. According to the Food and Agriculture Organization (FAO) yearbook production [2], an estimated 80% of the world's cattle are infested by ticks, giving rise to an annual cost of 7500 million US dollar. Host vaccination against tick infestation has been shown to be the most sustainable alternative tick control method to the current use of acaricides which has serious limitations such as contamination of livestock food products and quick development of tick resistance against acaricides [3]. The success of this method is dependent on the identification and characterization of tick molecules responsible for the biological success of the tick [4]. Induction of immediate hypersensitivity reaction by tick saliva proteins was linked to expression of acquired tick resistance by hosts undergoing secondary tick infestation [3–7]. Studies by Binta and Cunningham [8], Walker and Fletcher [9] and Willadsen et al. [10] demonstrated that the sensitivity of cattle to tick molecules inducing an immediate hypersensitivity re-

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action is directly correlated to the level of acquired anti-tick resistance by the host. Based on evidence from the above studies, it is interesting to examine the possibility that tick molecules inducing immediate hypersensitivity reactions in tick-primed hosts can confer protective anti-tick immunity in naive hosts. Any investigation of the immunological basis of resistance to ticks will be complicated if the only test material available is a crude tick extract containing a mixture of antigens. Recently the authors of this study used a combination of both immuno- and plaque hybridization screening of a cDNA library to clone and express in *Escherichia coli* an immunodominant 29 kDa (p29) salivary gland associated protein from *Haemaphysalis longicornis* [13]. Vaccination of naive rabbits with rp29 induced significant protective immunity resulting in 40 and 56% mortality of larvae and nymphal ticks as well as a significant reduction in engorgement weight of all adult ticks which were fed on rp29 immunized rabbits [13]. In order to enhance the rp29 induced immunity, it is necessary that other molecules are defined and used in cocktail with rp29. The objective of the present study was to attempt to purify and characterize tick saliva proteins capable of inducing an immediate hypersensitivity reaction in rabbits sensitized to *Haemaphysalis longicornis* tick feeding. *H. longicornis* is a three host tick commonly infesting cattle, dogs and humans and a vector of *Theileria* spp. as well as *Coxiella burnettii* [11].

2. Materials and methods

2.1. Preparation of whole tick extract (WTE)

H. longicornis larval ticks used in this study were obtained from a colony maintained on Japanese white rabbits in our laboratory. WTE was prepared by homogenizing unfed larval ticks in phosphate-buffered saline (PBS, pH 7.4, 10 mM sodium phosphate, 0.15 M sodium chloride) using a homogenizer (Polytron Kinematica AG, Switzerland) on ice. The homogenate was centrifuged and the collected supernatant (WTE) was stored at −80°C until used. Protein concentration was determined by using the BCA kit (Pierce Inc., USA). Due to technical problems associated with handling larval ticks, the number of ticks used to generate the starting crude homogenate was not determined.

2.2. Skin test (induction of immediate hypersensitivity reaction)

The skin test was carried out using Japanese white rabbits expressing an apparent acquired immunity against tick infestation. Acquired immunity to tick infestation was induced by repeatedly feeding ticks on rabbits up to four infestations. For skin testing, rabbits were shaved on the flanks and 3–10 ml of Evans blue dye (5 mg/ml) was injected intravenously in the ear vein using a 24 gauge needle. Within 10 min of injecting the dye, test sample aliquots with protein concentrations between 0.1 and 100 μg were injected intradermally into the shaved flanks. For positive and negative controls, an aliquot of the material loaded to the column or a blank (PBS or diluent of the test antigen) were used respectively. A positive skin test reaction representing induction of an immediate hypersensitivity reaction was recorded with appearance of a blue spot at the injection site of the test sample. The results were recorded within 15–30 min after injecting the antigen. During our preliminary skin test experiments, we had observed an increase of false positives when rabbits were shaved shortly before injecting the dye. To reduce false positives, rabbits were shaved 12–18 h prior to skin testing. Following the skin test, rabbits were rested for 1–2 weeks for the dye to be completely cleared before being reused. Three rabbits, two immune and one naive, were used for the skin test.

2.3. Antigen purification

The antigen was purified using a combination of ion exchange (IEC), reverse phase (RPC) and hydrophobic interaction (HIC) chromatographies. The larval tick extract prepared as described above was dialyzed against IEC starting buffer (20 mM Tris–HCl, pH 8.0) overnight at 4°C. Approximately 260–780 μg/run (2.6 mg/ml of total protein (10 mg)) of the dialyzed extract was loaded on to the IEC column (1 ml RESOURCE Q, Pharmacia, Sweden). The column was equilibrated and washed with starting buffer and materials bound to the column

were eluted with a 0–100% linear gradient of 0.5 M sodium chloride in the starting buffer at a flow rate of 1.0 ml/min. Protein elution was monitored by absorbance at 280 nm. The fractions were pooled per retention time and used in the skin test. The skin test positive fractions were concentrated by ultrafiltration using a Centrisart 1 spin-concentrator (Sartorious GmbH, Germany) with >10 kDa cut off point and subsequently subjected to RPC. The concentrated IEC fractions were dialyzed against distilled water (H_2O) overnight at 4°C prior to loading onto a RPC column (TSK gel-5 PW, Tosoh Co., Japan). The column was equilibrated and washed with 0.05% trifluoroacetic acid (TFA) diluted in H_2O. The bound proteins were eluted with a 20–100% linear gradient of 80% acetonitrile/H_2O (V/V)-0.05% TFA at a flow rate of 0.5 ml/min and fraction peaks were monitored at 215 nm. RPC fractions were concentrated by lyophilization under vacuum and subsequently reconstituted in H_2O before subjecting them to the skin test. A partially purified skin test positive fraction resulting from RPC was subjected to HIC using the RESOURCE PHE HIC column (Pharmacia, Sweden). The column was equilibrated and washed with HIC start buffer (50 mM sodium phosphate buffer, pH 7.0 containing 1.5 M $(NH_4)_2SO_4$) and eluted with a 0–100% linear gradient of elution buffer (50 mM sodium phosphate buffer, pH 7.0) with protein elution monitored at 280 nm absorbance. Routinely skin test positive fractions of every purification step were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5, 10 or 15% polyacrylamide gels essentially as described below.

2.4. SDS-PAGE-silver staining and immunoblot analysis

Protein analysis by SDS-PAGE was carried out in gels of 7.5,10 or 15% polyacrylamide gels essentially as described elsewhere [12]. Silver staining was done by using a kit according to the manufacturer's instructions (Daiichi, Japan). For Western blotting analysis, the purified polypeptide was electrophoresed on a 7.5% polyacrylamide gel and electroblotted to a polyvinylidene difluoride membrane (PVDF, Millipore, USA). The membrane was probed with polyclonal anti-tick serum collected from rabbits expressing apparent immunity against *H. longicornis* ticks. Positive signal detection was done with peroxidase-conjugated goat anti-rabbit IgG in 3,3-diaminobenzidine tetrahydrochloride and cobalt chloride diluted in PBS.

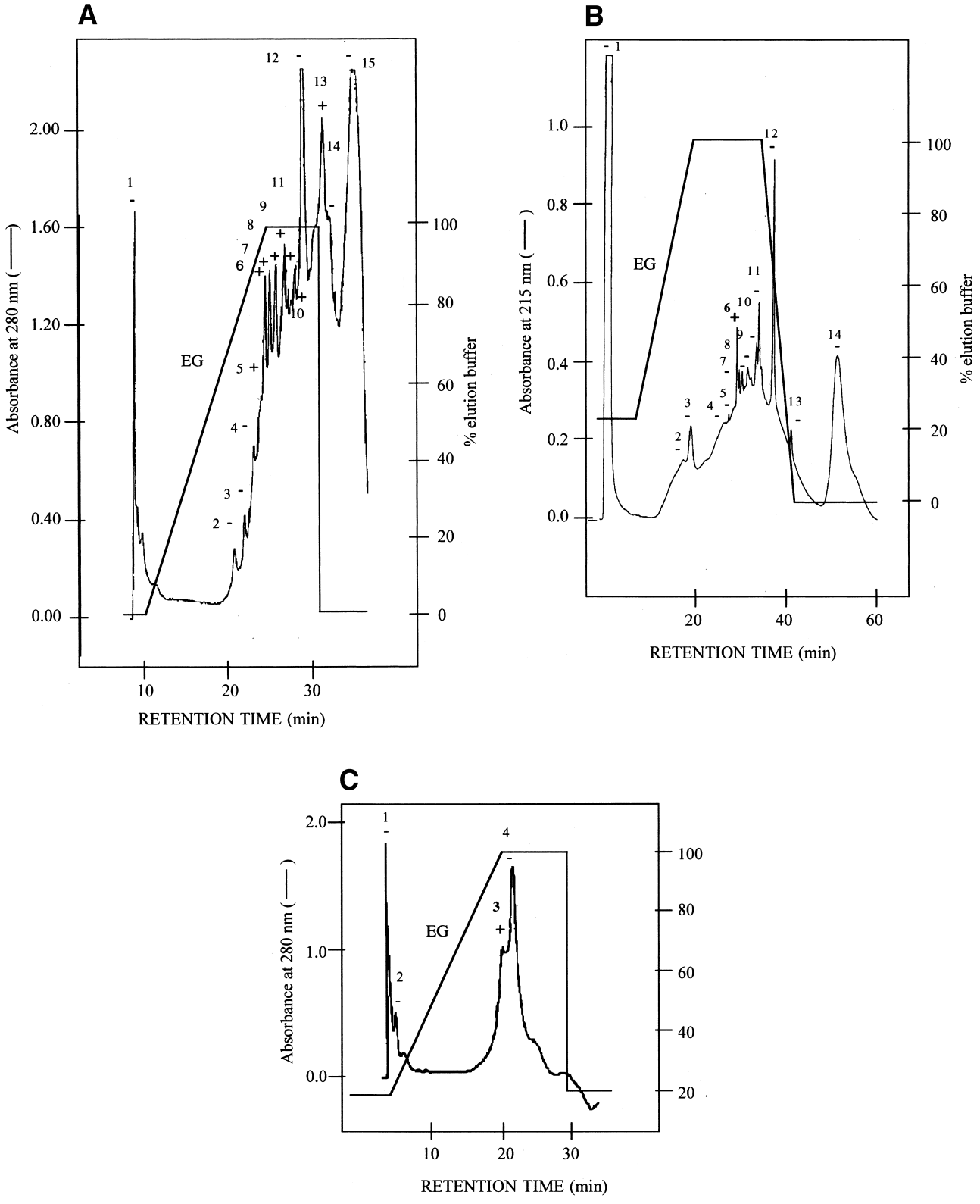
2.5. Amino acid sequencing

For N-terminal sequencing, 8 μg of the purified final product in H_2O was blotted onto the sequencing grade PVDF membranes (Immobilon-pSQ, Millipore, USA), dried at room temperature and applied to the automated amino acid sequencer (Applied Biosystems 476A, USA) according to the manufacturer's instructions. For internal amino acid sequencing, approximately 15 μg of the purified product in H_2O was lyophilized in a speed vacuum concentrator (Taiyo, Japan). The lyophilized product was reconstituted in 50 ml of 100 mM NH_4HCO_3 buffer, pH 9.0 and digested with 0.5 μg of modified porcine trypsin (Sequencing grade, Promega, USA) overnight at 37°C. The trypsin digests were applied to a reverse phase C18 column (Syncrom Inc, USA) for peptide isolation. The column was equilibrated with 0.06% TFA and eluted with a 10–60% linear gradient of 80% acetonitrile/0.06% TFA in H_2O at a flow rate of 0.06 ml/min. Peptide peaks were collected and subjected to automated sequencing (476A, Applied Biosystems, USA) according to the manufacturer's instructions. The obtained amino acid sequences were used to search the protein database for similarity comparisons with other known proteins.

3. Results

3.1. Antigen purification and Western blotting analysis

Induction of skin hypersensitivity reaction was indicated by a blueing reaction at the site of injection of the test material. Fig. 1A–C summarizes purification of the antigen, subsequently referred to in the text as p84. As shown in Fig. 1A, eight of the 15 IEC fractions were skin test positive and mostly eluted between 16 and 28 min of retention time. Following concentration by ultrafiltration and dialysis against water as described in Section 2, except IEC fraction


A
Absorbance at 280 nm (——)
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RETENTION TIME (min)
B
Absorbance at 215 nm (——)
% elution buffer
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RETENTION TIME (min)
C
Absorbance at 280 nm (——)
EG
RETENTION TIME (min)

Fig. 1. Antigen (p84) purification by IEC, RPC and HIC. The elution profile of (A) whole larval tick extracts applied to an ion exchange 1 ml RESOURCE Q column. Approximately 260–780 μg/run was loaded to the column per run. (B) IEC fraction number 7 subjected to RPC using the TSK-GEL 5PW column. (C) The partially purified skin test positive fraction resulting from RPC subjected to HIC using the RESOURCE PHE column. − = skin test negative fractions, + = skin test positive fractions. EG = elution gradient, fraction peaks are numbered.
←

no. 7, the other fractions did not yield any skin test positive fractions when applied to the RPC column.

Out of the 14 RPC fractions of IEC fraction no. 7 (Fig. 1B), fraction no. 6 which eluted at 29 min retention time was skin test positive. When subjected to SDS-PAGE and silver staining, the RPC skin test positive fraction showed a predominant 84 kDa band but appeared partially purified. Of the five HIC fractions shown in Fig. 1C, fraction no. 3 at 19 min retention time gave a skin test positive response when injected intradermally in rabbits expressing an apparent immunity against tick infestation but not in naive rabbits. SDS-PAGE and silver staining analysis of the skin test positive fraction showed a single 84 kDa (p84) band indicating that it had been purified to homogeneity (Fig. 2). Approximately 50 μg of

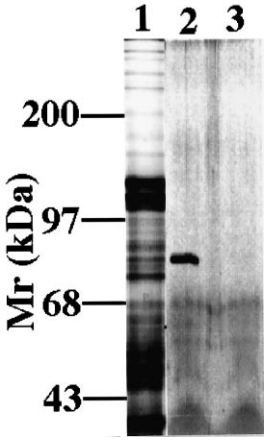

Fig. 2. SDS-PAGE analysis of purified p84. Discontinuous reducing SDS-PAGE and silver staining of the skin test positive, HIC fraction number 3. A portion of the HIC skin test positive fraction was mixed with 2×SDS-PAGE sample buffer and electrophoresed on a 7.5% acrylamide gel. Gel staining was done using a silver staining kit. M_r = molecular mass markers (Bio-Rad, UK), Lane 1 = crude tick homogenate, lane 2 = final product, lane 3 = empty.

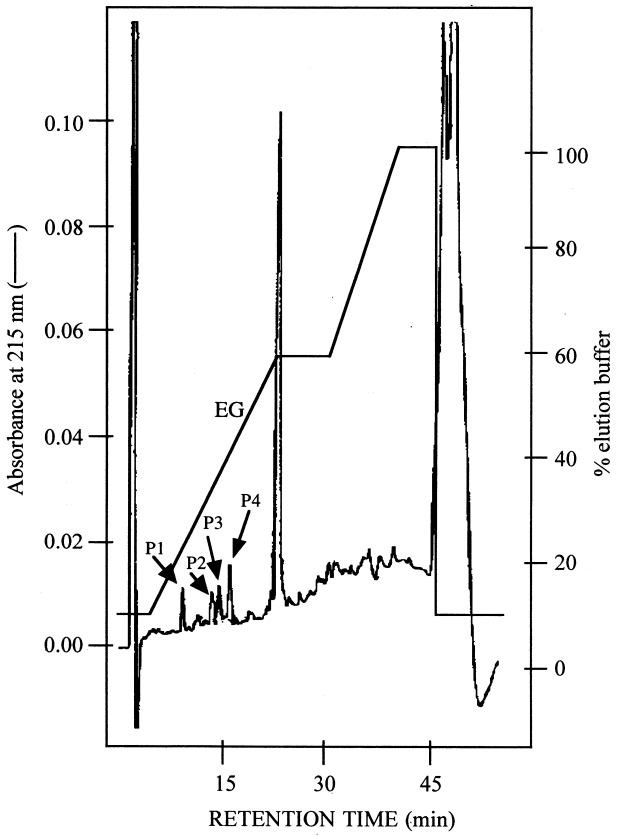

Fig. 3. Peptide isolation. Elution profile of the p84 protein trypsin digest applied to reverse phase HPLC C18 column for peptide isolation. P1–P4 = peptide fractions subsequently subjected to amino acid sequencing, EG = elution gradient.

purified p84 was obtained, representing 0.5% of the starting 10 mg of crude total protein. On the immunoblot of the purified p84 probed with rabbit anti-tick immune serum, no positive signal was detected with peroxidase-conjugated goat anti-rabbit IgG (result not shown).

3.2. *Amino acid sequencing and comparison to database proteins*

The N-terminal amino acid sequencing was insignificant possibly due to a N-terminal blocking. The purified p84 was subjected to an overnight trypsin digestion and peptides isolated as described in Section 2. As shown in Fig. 3, four peptide peaks were isolated and loaded to an automated amino acid sequencer. Peptides, P2 and P4 gave significant amino acid sequences shown in Fig. 4a,b, while peptides P1 and P3 did not yield any significant amino acid

a

P84 PEPTIDE P2	**ISGWGNT**YLI**TAS**
Porcine trypsin precursor	**ISGWGNT**KSSGS**S**
Bos taurus trypsin precursor	**ISGWGNT**KSSGT**S**
Xenopus laevis trypsin precursor	**ISGWGNT**ISNGSN
Human trypsinogen 1 precursor	**ISGWGNT**ASSG**A**D
Haematobia irritans serine protease	V**SGWGNT**KNPNED
Aedes aegypti trpsin 1 precursor	V**SGWGNT**QNPSE**S**
Anopheles gambaie trypsin 1 precursor	V**SGWGNT**QSAAE**S**
Anopheles gambaie trypsin 2 precursor	V**SGWGNT**QSAVE**S**
Salmon salsa trypsinogen precursor	V**SGWGNT**MSS**TA**.

b

P84 PEPTIDE P4	**HVPAGQI**TSNCHG
Escherichia coli ATP binding protein	**HVPAGQI**YGVIGA

Fig. 4. Similarity comparison of P2 and P4 amino acid sequences to known proteins on the SWISS-PROT database. The amino acid sequences P2 (a) and P4 (b) were used to search the SWISS-PROT database for homology to known proteins. Identical residues are boldface.

peaks. Based on the amino acid repetitive yields, >13.3 pmol of P2 and >10.6 pmol of P4 were subjected to sequencing. On comparison of these peptide amino acid sequences to known proteins on the SWISS-PROT database, a 7 residue motif, ISGWGNT present in P2 appeared conserved among all known trypsin-like serine proteinases from both vertebrates and invertebrates (Fig. 4a). Additionally, as shown in Fig. 4b, a 7 residue motif, HVPAGQI present in P4 showed homology to an ATP-binding protein of *E. coli*.

4. Discussion

The present study describes purification and characterization of an 84 kDa trypsin-like serine proteinase capable of inducing an immediate hypersensitivity reaction in rabbits sensitized to tick feeding. The final product was purified to homogeneity as shown by SDS-PAGE and silver staining analysis. Following ultrafiltration on a membrane with >10 kDa cut off point, the majority of the skin test positive peaks from IEC did not yield any skin test positive fractions when subjected to RPC. This may suggest that the molecular mass of the active molecules in these fractions could have been less than 10 kDa and thus were filtered out during the concentration step. Additionally the active molecules which eluted the IEC column could have been irreversibly bound to the RPC column and hence did not elute. While available data may be insufficient to make a definitive conclusion, evidence from comparison of the obtained peptide amino acid sequence to protein sequences on the database may suggest that the purified p84 reported in this study possibly belongs to a class trypsin-like serine proteinases. The serine proteinase family is one of the most conserved class of proteins across many species and involved in mediation of several key physiological functions such as the vertebrate coagulation cascade, nutrition, fertilization and development regulation [13,14]. Despite the lack of baseline information with regard to the role of serine proteinases in the biological success of ticks both as vectors and pests, there is abundant compelling indirect evidence that makes members of the serine proteinase gene family appealing as target antigens for a tick vaccine. For example, addition of serine protease inhibitors such as soybean trypsin to a blood meal inhibited egg production up to 71% in *Stomoxys calcitrans* [15]. In a related study, Elvin et al. [16] have indicated that inclusion of low levels of serine protease inhibitors such as soybean trypsin or ovomucoid in the blood meal can either kill *Haematobia irritans* or inhibit egg development, while Casu et al. [17] have also observed a reduced growth rate of *Lucilia cuprina* larvae when they were fed in vitro with soybean trypsin inhibitor. Vaccination of cattle against *Hypoderma lineatum* with a purified serine protease stimulated a strong cellular and humoral immunity leading to 95% protection of cattle against *H. lineatum* infestation [18]. In another study Bowles et al. [19] showed that host plasma protease inhibitors can reduce the growth rate of sheep blowfly, *L. cuprina* larvae in vitro, suggesting that the secretory/excretory proteinases of *L. cuprina* may be of special significance in initiating wounds. The possibility that P2 is derived from porcine trypsin which was used for digestion of p84 is ruled out, because after the conserved 7 residue motif, the P2

amino acid sequence had very few or no identical residues to porcine trypsin or any of the other mammalian trypsins. It will be interesting to obtain the entire primary structure of p84 in order to establish its identity.

Similar molecules capable of inducing immediate hypersensitivity reaction in tick resistant hosts have been purified from other ticks species. Physiochemical approaches were used to identify and purify four antigens from *Boophilus microplus* extracts including a 30 kDa, an esterase with carbohydrate splitting activity [20], 60 kDa allergen 1, an esterase [21], 24 kDa allergen 2 [10] and an 18 kDa, a trypsin enzyme inhibitor [22]. In another study, Gill et al. [23], isolated from *Hyalomma analoticum analoticum* three glycoproteins antigens I (130 kDa), II (103 kDa) and III (96 kDa) which were reacted by anti-*H. analoticum* rabbit immune serum on immunoblots and induced skin hypersensitivity reaction in tick resistant rabbits [24–26].

The fact that the purified product did not induce skin hypersensitivity reaction in rabbits naive to tick infestation indicated that p84 was specifically recognized as an immunogen in rabbits that were primed against tick feeding. However no positive signal was detected on p84 blots when probed with rabbit anti-tick immune IgG which may suggest that p84 is capable of inducing the skin hypersensitivity serum but not IgG production. Studies to clone and express the p84 cDNA in vitro are in progress in order to elucidate its (p84) biological function and its possible use as a tick vaccine candidate.

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